

Antiproteinase chemotherapy of acute experimental pancreatitis using the low molecular weight oligopeptide aldehyde leupeptin

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SUMMARY Continuous intravenous infusion of the low molecular weight trypsin inhibitor leupeptin prolonged the survival of rats with acute haemorrhagic pancreatitis ($p < 0.001$) compared with controls receiving saline alone. Rats receiving high dose intravenous Trasylol (aprotinin) survived no longer than saline-only controls. Combination therapy of leupeptin with Trasylol conferred no additional benefit over animals treated with leupeptin alone. The nature of the infusion was selected blind after the induction of pancreatitis and survival was quantified by recording of body temperature. These preliminary results suggest that sterically favourable molecules which can complete the inhibition of α_2 -macroglobulin bound proteinases should contribute to the effective specific chemotherapy of the disease.

It is generally accepted that a crucial event in the pathogenesis of acute necrotising pancreatitis is the overwhelming and inappropriate activation of digestive zymogens within the gland leading to the autodigestion of pancreatic tissue and the massive dissemination of active enzymes.¹⁻⁵ In health such events are prevented by the biosynthesis of inactive precursor forms of the most dangerous enzymes and their storage in membrane-bound granules within the acinar cells. Release of these zymogens into the pancreatic ducts and their subsequent activation only within the gut lumen is a further safeguard. The acinar cell also produces trypsin inhibitors which complex with active free trypsin preventing the potentially disastrous activation of the other proenzymes.^{1,6} If free trypsin, chymotrypsin, and elastase are released into blood they immediately complex with the serum proteinase inhibitors, α_1 -antiproteinase and α_2 -macroglobulin.⁷⁻¹⁰ The α_1 -antiproteinase-enzyme complex is catalytically inert but the α_2 -macroglobulin-enzyme complex retains a proportion of esterolytic and proteolytic activity against substrates of varying molecular size.¹¹⁻¹³ Normally these complexes are rapidly eliminated from the circulation by the reticuloendothelial

system and circulating tryptic activity is insignificant.^{14,15} In severe acute pancreatitis the capacity to remove the catalytic complexes is exceeded;¹⁶ necrosis of pancreatic parenchyma, haemorrhage, and metastatic proteolytic damage occur and are associated with a high mortality.¹⁷

We have recently obtained good evidence to suggest that the reason that the protein antiproteinase Trasylol (aprotinin, K_i for free trypsin 3×10^{-11} M) is ineffective in acute necrotising pancreatitis is because the molecule is both too large (6400 daltons) and too inflexible to inhibit the catalytic activity of α_2 -macroglobulin bound proteinases against biologically important substrates.¹⁸ Inhibition of these catalytic complexes was, however, completed by the low molecular weight oligopeptide aldehydes leupeptin (474 daltons) and antipain (674 daltons); this suggests that these inhibitors might contribute effectively to the specific chemotherapy of acute necrotising pancreatitis.¹⁸ Leupeptin and antipain belong to a series of oligopeptide proteinase inhibitors produced by specific strains of streptomycetes.¹⁹ They have been isolated and characterised by Umezawa and co-workers^{20,21} and shown to be potent competitive inhibitors of many enzymes including trypsin, thrombin, plasmin, some lysosomal hydrolases, and papain. This paper reports the effect of a partly purified preparation of one of these oligopeptide inhibitors, leupeptin, on the course of

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acute haemorrhagic pancreatitis in rats and compares the results with those obtained using the protein antiproteinase Trasylol.

Methods

Leupeptin was kindly provided by Professor H Umezawa, Institute of Microbial Chemistry, Tokyo, Japan; sodium taurocholate was obtained from BDH Chemicals, Poole, Dorset, UK, and used without further purification. 2x-crystallised bovine trypsin was from Sigma Chemicals, Poole, Dorset, UK. Trasylol was from Bayer (UK) Hayward's Heath, Sussex, and Biogel P2 was from Bio-Rad Laboratories, Watford, Herts, UK.

CHROMATOGRAPHY AND TOXICITY TESTING OF LEUPEPTIN

Two hundred milligram aliquots of the partly purified preparation of leupeptin were solubilised in 1 ml 50 mM acetic acid and fractionated on a 185 ml bed of Biogel P2. The column eluate was monitored at 220 nm and the material was divided into four fractions, lyophilised, and characterised by thin layer chromatography.²⁰ Each fraction was tested for acute toxicity *in vivo*.

Two hundred and fifty gram male Wistar Firth rats were anaesthetised with intraperitoneal pentobarbitone sodium (6 mg/100 g body weight) and the left femoral artery and vein were cannulated. The arterial line was connected to a pressure transducer and the blood pressure was monitored on a recorder; respiration was also observed. Aliquots of the four leupeptin fractions varying from 2 mg/kg to 20 mg/kg body weight were administered as single doses intravenously over a one minute period.

STANDARDISED INDUCTION OF ACUTE PANCREATITIS IN RATS

A modification of Lankisch's method²² was used in the induction of acute pancreatitis. Male Wistar Firth rats weighing between 230–270 g were anaesthetised with intraperitoneal pentobarbitone sodium (6 mg/100 g body weight) and the duodenum delivered through a midline abdominal incision. The proximal end of the common bile duct was occluded by a 8×1.5 mm Codman straight neurosurgical aneurysm clip (Hamblin Instruments, London) and the distal end cannulated by transduodenal puncture with a 22 gauge Argyll medicut Teflon cannula, held in place by an encircling suture. A standardised intraduct infusion of 2 ml per kg body weight of 3.5% (w/v) sodium taurocholate containing 6000 BAE units of trypsin per ml of isotonic saline and 2 mM CaCl₂ was delivered from a syringe drive pump at 0.05 ml/min. Techniques were developed to

permit continuous intravenous infusion²³ and body temperature monitoring in the unrestrained animals.²⁴ From this record the time of death could be precisely identified by the exponential fall in body temperature.

INTRAVENOUS INFUSIONS AND QUANTIFICATION OF SURVIVAL

In group A 15 rats received an intravenous dose of 9000 units of Trasylol on wound closure followed by 3500 units/h at an infusion rate of 1 ml/h; 15 control animals received an equal volume of saline alone. In group B 15 rats received an intravenous dose of 0.2 mg of the fractionated leupeptin before an hourly infusion of 0.2 mg of the same material; an additional 15 rats received saline alone. In group C 15 rats were given 9000 units of Trasylol together with 0.2 mg leupeptin on wound closure followed by an hourly infusion of 3500 units of Trasylol and 0.2 mg leupeptin; 15 animals received leupeptin alone on an identical dosage regime to those in group B; and 15 control animals again received equivalent volumes of saline alone. At the time of induction of pancreatitis the nature of the intravenous infusion (test or control) was not known and was selected subsequently for each animal by drawing a coloured disc. The presence or absence of therapeutic effect was based upon duration of survival measured on the trace to the nearest hour. Survival times were compared using a two sample Student's *t* test.

Results

FRACTIONATION AND TOXICITY TESTING OF LEUPEPTIN

The elution profile of partly purified leupeptin on Biogel P2 chromatography is shown in Fig. 1. Of the four pooled fractions I, II, and III were toxic to rats; I had an LD₅₀ of ~10 mg/kg body weight, and fractions II and III had an LD₅₀ of ~20 mg/kg body weight when given as an intravenous dose over a one minute period. The animals died within two minutes of administration from simultaneous apnoea and severe hypotension. Fractions II and III contained the anti-trypsin activity (K_i ~1.25 μ M) and were combined and used for subsequent *in vivo* studies. Thin layer chromatographic analysis consistently showed two spots at the predicted R_f values for acetyl- and propionyl-leupeptin.²⁰

SURVIVAL AFTER INDUCTION OF PANCREATITIS

Figure 2a shows that Trasylol had no beneficial effect on survival in acute pancreatitis compared with the continuous intravenous infusion of saline alone (group A). Mean survival time in hours \pm ISD was 25.4 \pm 10.5 for the saline group and 25.7 \pm 9.9 for

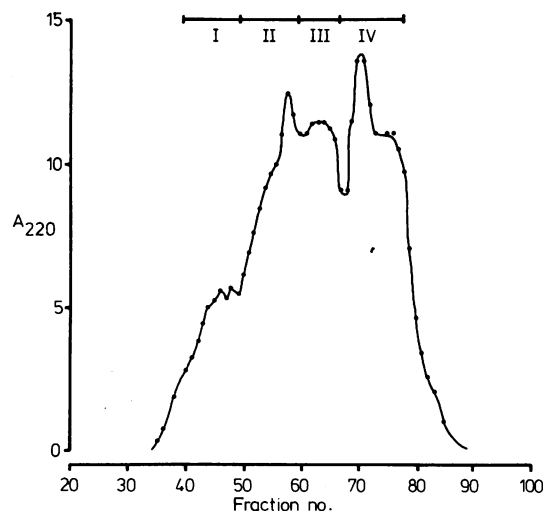


Fig. 1 Elution profile of partly purified leupeptin after chromatography on a column of Biogel P2 (2.54×37 cm) 2 ml fractions pooled I–VI as indicated. For details see text.

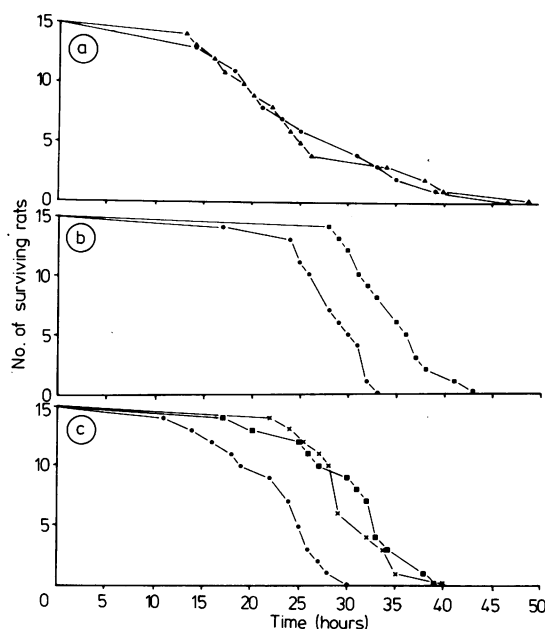


Fig. 2 Survival times of rats with acute pancreatitis receiving continuous intravenous infusions of either (a) Trasyolol (▲—▲) or saline (●—●), (b) leupeptin (■—■) or saline, (c) leupeptin with Trasyolol (x—x), leupeptin alone, or saline. For dosages see Methods section.

the Trasyolol group. Figure 2b shows that rats receiving continuous intravenous leupeptin as described survived longer than those receiving saline alone (group B). Mean survival time in hours \pm ISD was 28.0 ± 4.2 for the saline controls and 34.4 ± 4.4 for the leupeptin treated animals; this difference was statistically significant ($p < 0.001$). No additional benefit was conferred by including Trasyolol in the leupeptin infusion (group C) (Fig. 2c). Mean survival time in hours \pm ISD were 22.3 ± 5.5 for the control rats, 30.0 ± 4.8 ($p < 0.001$) for the combined leupeptin/Trasyolol treated animals, and 30.4 ± 6.4 ($p < 0.001$) for the rats receiving leupeptin alone. There was no significant difference in group C between the rats receiving the combination therapy of leupeptin and Trasyolol and those just receiving leupeptin ($p > 0.5$).

Discussion

The method described here for the standardised induction of acute haemorrhagic pancreatitis is a modification of that described by Lankisch *et al.*²² Accurate, automated monitoring of survival times revealed, in preliminary studies, the poor reproducibility associated with manual intraductal injection. This was not apparent when only the 24-hour survivors were scored.²² Therefore mechanised, continuous, slow intraductal infusion was developed which, within each group, provided good reproducibility. Because of the inherently variable susceptibilities of any experimental animal, particularly over the duration of this type of study, care was taken to match equal numbers of control and treated animals on the same day.

Many specific treatments have been tried in acute pancreatitis with two principal objectives: either the inhibition or removal of disseminated active enzymes or the prevention of further zymogen biosynthesis and release by pancreatic acinar cells.^{17 25–31} Therapies described in the former category include peritoneal lavage¹⁷ and the administration of antiproteases,^{25 26} whereas anticholinergics,²⁷ glucagon,²⁶ calcitonin,²⁸ and somatostatin²⁹ are in the latter group. In all cases results have at best been equivocal and no therapy has been demonstrated to consistently reduce morbidity or mortality of the disease. Metastatic proteolytic damage by disseminated α_2 -macroglobulin-pancreatic-proteinase complexes in acute necrotising pancreatitis will be maximal in the early hours of the disease when mortality is highest and the need for effective specific treatment is most urgent. Three recent randomised clinical trials have shown that Trasyolol fails to confer benefit over standard conservative management of patients with

acute pancreatitis.^{25 26 32} This is most likely because of the inability of Trasylol to inhibit the activity of trypsin complexed with α_2 -macroglobulin due to its failure to gain access to the shielded catalytic site.¹⁸ Susceptible peptide hormones such as glucagon and somatostatin, infused during this period of severe acute pancreatitis will be rapidly degraded by catalytic α_2 -macroglobulin enzyme complexes against which Trasylol will afford little protection. The use of low-molecular weight anti-proteinase would appear to be advantageous, as they should be independent of these steric constraints.

In this study Trasylol was without effect in prolonging the survival of rats with experimental pancreatitis despite its early administration and continuous intravenous infusion at 5 \times the recommended clinical dose for man. Intravenous leupeptin resulted in an extension of survival; although this effect was statistically significant, none of the leupeptin treated rats eventually survived. Further studies using highly purified leupeptin are clearly indicated, as residual toxic components may have contributed to mortality in the leupeptin treated animals and certainly limited the dose of the inhibitor that could be used, but these are preliminary findings and form the basis of a continuing study into the use of peptide aldehyde protease inhibitors in the treatment of acute pancreatitis. We would suggest that a combination of different low molecular weight inhibitors, either of bacterial origin or custom synthesised, with a wide spectrum of specificities for the digestive proteases could become an effective treatment for this disease.

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